PARTIAL PURIFICATION OF CYCLODEXTRIN GLUCANOTRANSFERASE FROM ALKALOPHILIC *Bacillus* sp. TS1-1

MOHD AZIM ABDUL RAHMAN

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DECLARATION

I declare that this thesis entitled “Partial Purification of Cyclodextrin Glucanotransferase from Alkalophilic *Bacillus* sp. TS1-1” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

Signature	:	…….
Name	:	Mohd Azim Abdul Rahman
Date	:	May 2008

DEDICATION

Special dedication to
Khadijah Mustamar my beloved mother and
Abdul Rahman Mohd Seth my beloved father, both of you is everything to me
Baitilalia Ayu Zakaria, I believe in you and me

My siblings that always love me
My coursemates those always are challenging and supporting me

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ABSTRACT

The cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) from *Bacillus* sp. TS1-1 has been partially purified from crude culture by centrifugation and cross-flow filtration. Initial 72.96 U/ml of CGTase was detected in the culture after 24 hours of incubation. The crude supernatant obtained after centrifugation for 5000 rpm, 5 minutes and 4°C was subsequently filtered at 15°C through cross-flow filtration using Kvick Lab cross-flow system. Two cassettes were used with molecular weight cut off of 50K and 10K. The retentate from 50K cassette was further filtered through 10K cassette. Each permeate and retentate from each cassette were tested for CGTase activity. The highest CGTase activity was detected at retentate of 10K cassette which suggested an initial size of CGTase between 10 kDa and 50 kDa. The crude and partially purified enzyme was then subjected to electrophoresis (SDS-PAGE). It was clear that a large amount of proteins had been removed by cross-flow filtration. Based on this work, the crude enzyme was purified 5.57 fold using cross-flow filtration.

ABSTRAK

Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) daripada *Bacillus* sp. TS1-1 telah ditulenkan secara separa daripada kultur mentah menggunakan kaedah pengemparan dan penyaringan aliran melintang. Sebanyak 72.96 U/ml daripada CGTase telah dikesan di dalam kultur selepas 24 jam inkubasi. Supernatan mentah yang diperolehi selepas pengemparan pada 5000 rpm, 5 minit and 4°C yang kemudiannya disaring pada 15°C melalui penyaringan aliran melintang menggunakan sistem penyaring Kvick Lab. Dua blok penyaring yang telah digunakan ialah bersaiz rongga 50K dan 10K. Baki daripada penyaring 50K seterusnya disaring melalui penyaring 10K. Setiap hasil dan baki daripada setiap blok penyaring kemudian diuji untuk aktiviti CGTase. Aktiviti CGTase yang paling tinggi dikesan pada baki penyaring 10K yang mungkin menunjukkan saiz awal CGTase antara 10 kDa dan 50 kDa. Enzim mentah dan enzim yang telah ditulenkan secara separa kemudiannya dianalisis dengan elektroforesis (SDS-PAGE). Adalah jelas bahawa sejumlah besar protein telah disingkirkan semasa penyaringan aliran melintang. Berdasarkan kajian ini, enzim mentah telah ditulenkan sebanyak 5.57 kali ganda menggunakan penyaringan aliran melintang.

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Å	-	angstrom
ADP	-	adenosine diphosphate
ATP	-	adenosine triphosphate
CD	-	cyclodextrin
CGTase	-	cyclodextrin glucanotransferase
h	-	hour
K	-	kilo Dalton (molecular weight cut off)
kDa	-	kilo Dalton
mg	-	milligram
min	-	minute
mM	-	millimolar
mol wt	-	molecular weight
µl	-	microliter
µmol	-	micromole
N	-	number of mole
ng	-	nanogram
nm	-	nanometer
OD	-	optical density
pI	-	isoelectric point
rpm	-	revolution per minute
SDS-PAGE	-	sodium dodecyl sulphate polyacrylamide gel
U	-	unit (enzyme activity)
UV	-	ultraviolet
V	-	volt
v/v	-	volume per volume
w/v	-	weight per volume

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) are able to convert starch into cyclodextrins (CDs), closed-ring structures in which six or more glucose units are joined by means of α-1,4 glycosidic bonds (Hashimoto, 1988). This enzyme is an important enzyme in industries of food, analytical chemistry, agriculture, pharmaceutical field, and toilet articles. Generally, CDs exist as three different types: α-CD, β-CD and γ-CD, containing six, seven and eight glucose residues, respectively.

Villiers first observed CDs in 1891 when in addition to reducing dextrins a small amount of crystalline material was obtained from starch degrades of *Bacillus amylobacter*. The main interest in cyclodextrins lies in their ability to form inclusion complexes with several compounds. From the X-ray structures it appears that in cyclodextrins the secondary hydroxyl-groups (C2 and C3) are located on the wider edge of the ring and the primary hydroxyl-groups (C6) on the other edge, and that the apolar C3 and C5 hydrogens and the ether oxygens are at the inside of the torus-like molecules. This results in a molecule with a hydrophilic outside, which can dissolve in water, and an apolar cavity, which provides a hydrophobic matrix, described as a "micro heterogeneous environment" (Saenger, 1980).

CGTase are produced by many microorganisms. *Bacillus circulans* (Hofmann *et al*., 1989), *Bacillus firmus* (Shin *et al*., 2000), *Bacillus ohbensis* (Nishida *et al*., 1997), *Bacillus macerans* (Han *et al*., 1999), *Bacillus stearothermophilus* (Stefanova *et al*., 1999), *Klebsiella pneumoniae* (Gawande *et al*., 2001), *Bacillus* sp. TS1-1 (Mahat, 2004) and *Bacillus* sp. G1 (Sian *et al*., 2005) have been recognized as CGTase producer.

At present at least 38 CGTase enzymes have been identified and purified, and the matching genes cloned, mainly from *Bacillus* species, but also from *Thermoanaerobacterium, Thermoanaerobacter, Micrococcus* species, and from *Klebsiella*, a single Gram-negative source.

1.2 Problem Statement

The isolation of pure enzyme also allows active site studies to be carried out on the homogeneous protein, the characteristics of the enzyme such as kinetic parameters, optimum temperature and pH, temperature and pH stability, the effects of ions and crystallization of the enzyme for X-ray crystallographic analysis.

1.3 Objective

The objective of this research is to partially purify CGTase from alkalophilic *Bacillus* sp. TS1-1 that was isolated from soil.

1.4 Scopes

The scopes of this study are as follows

i. To produce crude CGTase from *Bacillus* sp. TS1-1
ii. To partially purify CGTase by cross-flow filtration
iii. To determine the degree of CGTase purification by SDS-PAGE

CHAPTER 2

LITERATURE REVIEW

2.1 Starch

2.1.1 Structure of starch

Starch has two types of glucan polymers: amylose and amylopectin. Potato starch as example consists of 20 % amylose and 80 % amylopectin. Depending on the origin, plant species, variety within plants, plant organ, age of organ, and growth conditions, this ratio may vary considerably, from 11 to 51 % amylose. Amylose consists mostly of linear chains of α-1,4 linked glucose residues, about 1000 residues long, and branched at a low level (about one branch per 1000 residues) by α-1,6 linkages. Pure amylose forms hydrogen bonds linking the molecules in solution, resulting in rigid gels. After heating this solution it might crystallize and shrink, this process known as retrogradation. The other 80 % of potato starch consists of amylopectin.

Amylopectin is a highly branched α-1,4 glucan polymer (approximately one α-1,6 linkage per 20 glucose residues), which forms organized structures (Figure 2.3). In these structures one can recognize the so called A-chains, which are not substituted at the C6 positions, the inner B-chains which are α-1,6 branched at one (B1-chain), or several points (B2, B3 etc.). There is only one free reducing end per amylopectin

molecule (the C-chain). The branches are clustered at 7-10 nm intervals (approximately 20 glucose residues), 20-40 per molecule, forming an amylopectin molecule 200-400 nm long (approximately 400-800 glucose residues long) and 15 nm wide. In solution amylopectin forms fewer hydrogen bonds than amylose, therefore it remains fluid, with a high viscosity and elasticity (Martin and Smith, 1995).

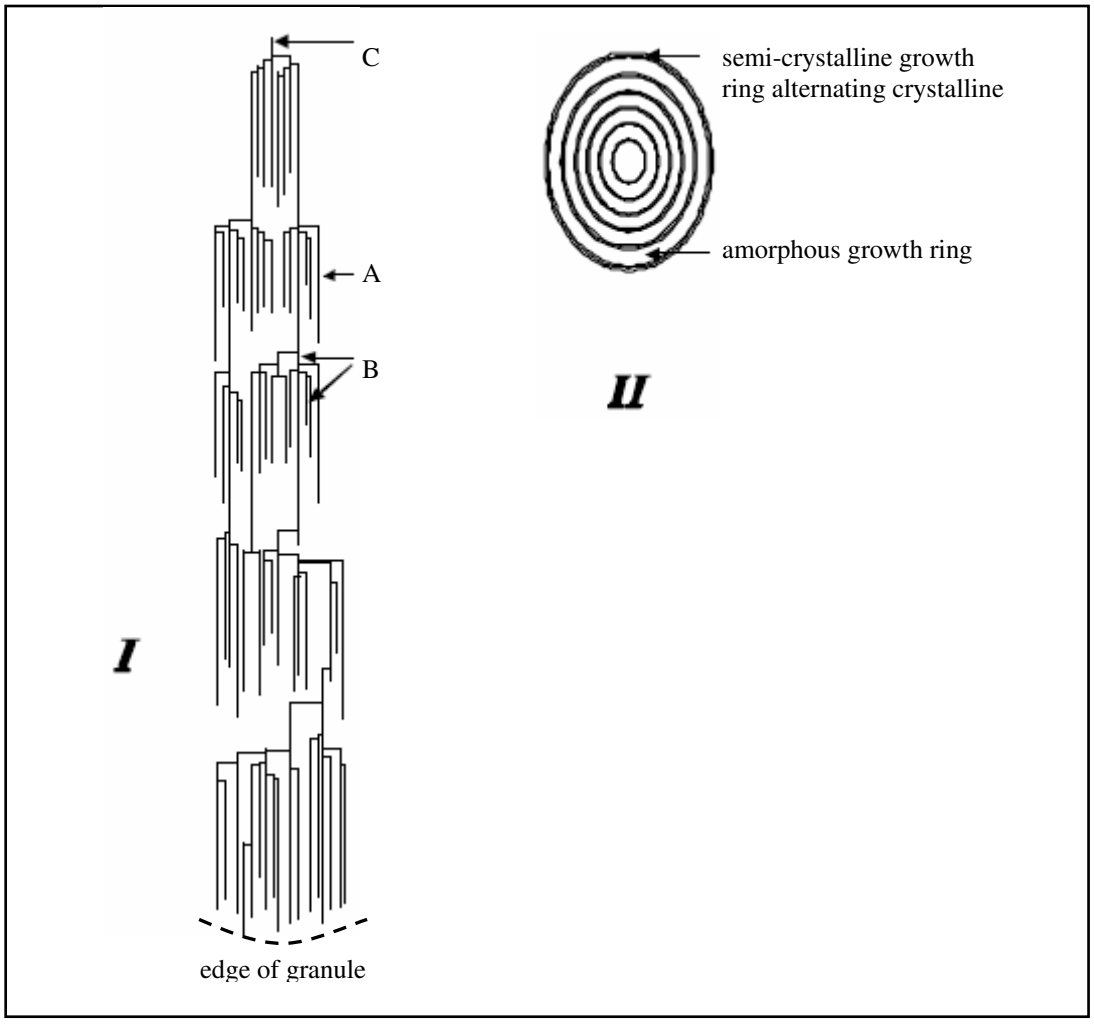

Figure 2.1: Schematic representation of starch.

I: Amylopectin molecule. Indicated are the A-, B-, and C-chains. A-chains are not branched; B-chains contain one branch (B1-chain) or more (B2-, B3-chain, etc). C-chains contain the single reducing end.

II: Starch granule. Show the alternating crystalline and amorphous growth rings.

(Martin and Smith, 1995)

2.1.2 The degradation of starch

The enzymatic process of starch biosynthesis is relatively simple; first an ADP-glucose pyrophosphorylase catalyzes the formation of an ADP-glucose and inorganic pyrophosphate from glucose-1-phosphate and ATP. For potato this takes place in the plant plastids (amyloplasts). Both ATP and glucose-1-phosphate have to be imported from the cytosol. Alternatively, glucose-1-phosphate can be synthesized in the plastid by a phosphoglucomutase from glucose-6-phosphate. The pyrophosphate is removed by the action of an alkaline pyrophosphatase.

The second step involves starch synthase, which catalyzes the formation of an α-1,4 bond between the C1 of the glucose from the ADP glucose and the C4 of the non-reducing glucose of the growing amylose chain. This suggests that an initial primer is needed to start the reaction; the nature of this primer is unknown.

A third step is formation of an α-1,6 branch by a starch-branching enzyme, which cuts an α-1,4 linked glucan chain and forms an α-1,6 linkage between the C1 at the reducing end of a released glucan chain and the C6 of a glucose residue in another chain. Branches are not created randomly, but show a 20 glucan residues periodicity. It is believed that this is due to the fact that the starch-branching enzyme has high affinity for a double helical conformation of glucan chains, which is only formed at a certain minimum chain length (Martin and Smith, 1995).

2.2 Cyclodextrin Glucanotransferase

Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) cleaves α-1,4 bonds in a starch molecule, concomitantly linking the reducing and non-reducing ends to produce a cyclic molecule. When starch is used as a carbon source of the organism, CGTase

converts starch into cyclodextrins, which are subsequently degraded by the action of the enzyme cyclodextrinase (Saha and Zeikus, 1990), which is associated with the membrane and is located at the cytosolic site. CGTase is excreted by the bacterium and produces a cyclodextrin (CD) from an amylose chain. A cell-associated CDase converts cyclodextrins into glucose and other oligosaccharides. Glucose (Glu) is subsequently used in glycolysis to produce pyruvate (Pyr) and ATP (Nakamura *et al*., 1993).

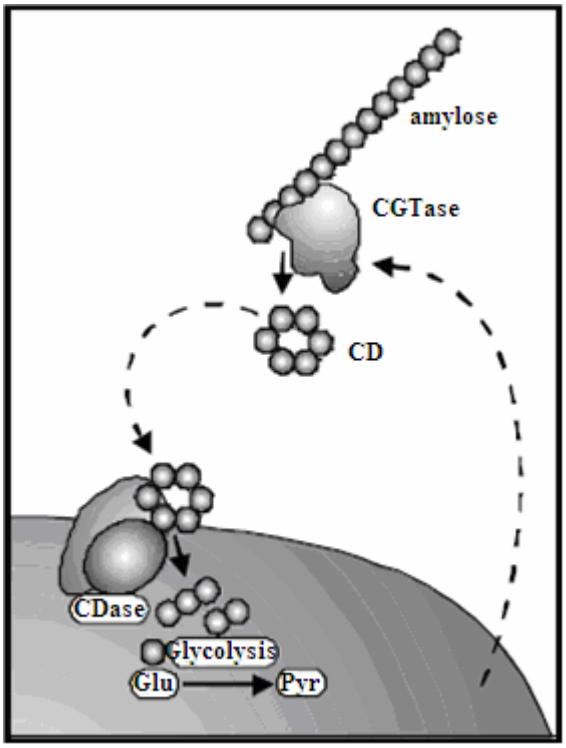

Figure 2.2: Schematic representation of the location and action of CGTase and CDase. Small circles indicate glucose residues (Nakamura *et al*., 1993)

CGTase are enzymes capable of several transferase reactions, in which a newly made reducing end of an oligosaccharide is transferred to an acceptor molecule. Depending on the nature of the acceptor molecule, four transferase reactions (cyclization, coupling, disproportionation, and hydrolysis) can be distinguished (Figure 2.3). Cyclization is the transfer of the reducing end sugar to another sugar residue in the same oligosaccharide chain, resulting in formation of a cyclic compound. Coupling is the reaction where a cyclodextrin molecule is combined with a linear oligosaccharide

(chain) to produce a longer chain linear oligosaccharide. Disproportionation is the transfer of part of a linear oligosaccharide chain to another linear acceptor chain. Starting from two molecules of a pure oligosaccharide, this reaction yields a mixture of smaller and longer oligosaccharides. In hydrolysis (saccharifying activity) the newly made reducing end is transferred to water (Nakamura *et al*., 1993).

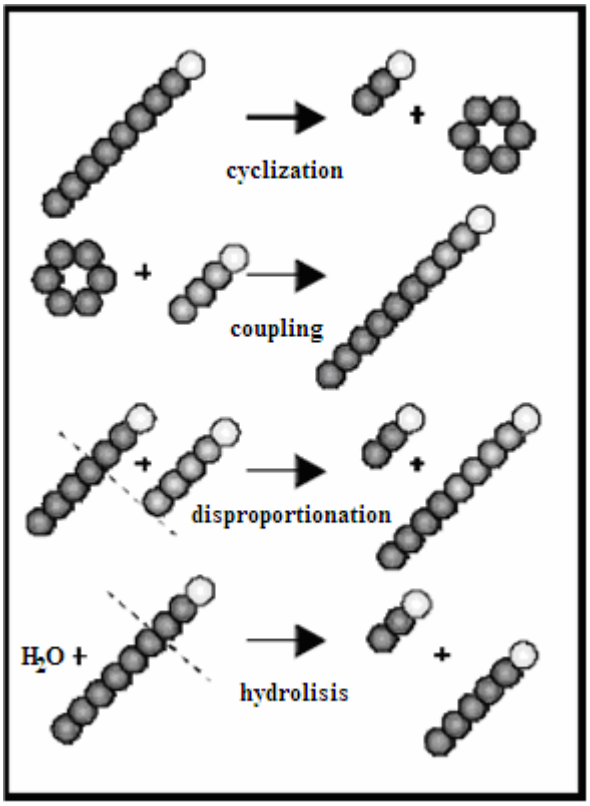

Figure 2.3: Schematic representation of the different activities of CGTase (Nakamura *et al*., 1993).

2.3 Cyclodextrins

2.3.1 Structure of cyclodextrins

Cyclodextrins are cyclic oligosaccharides consisting of six (α-cyclodextrin), seven (β-cyclodextrin), eight (γ-cyclodextrin) or more glucopyranose units linked by α-1,4 bonds (Figure 2.4). They were first discovered in 1891 when in addition to reducing dextrins a small amount of crystalline material was obtained from starch digest of *Bacillus amylobacter* (Villiers, 1891). According to other authors, Villiers probably used impure cultures and the cyclodextrins were produced by a *Bacillus macerans* contamination (Koch, 1891).

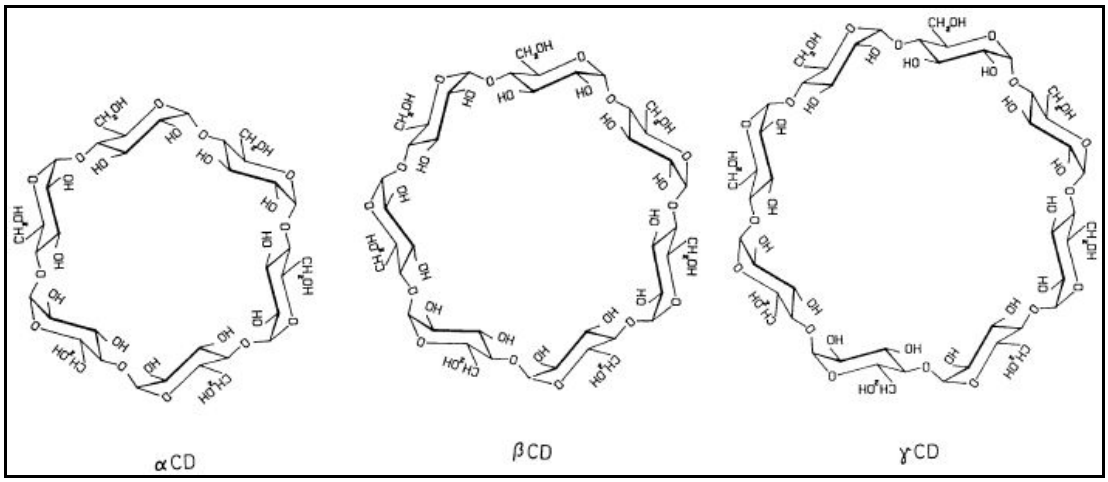

Figure 2.4: The structures of α-, β-, and γ-cyclodextrin (Szejtli, 2004)

The main interest in cyclodextrins lies in their ability to form inclusion complexes with several compounds. From the X-ray structures it appears that in cyclodextrins the secondary hydroxyl-groups (C2 and C3) are located on the wider edge of the ring and the primary hydroxyl-groups (C6) on the other edge, and that the apolar C3 and C5 hydrogens and ether like oxygens are at the inside of the torus-like molecules. This results in a molecule with a hydrophilic outside, which can dissolve in

water, and an apolar cavity, which provides a hydrophobic matrix, described as a "micro heterogeneous environment" (Saenger, 1980).

As a result of this cavity, cyclodextrins are able to form inclusion complexes with a wide variety of hydrophobic guest molecules (Figure 2.5). One or two guest molecules can be entrapped by one, two or three cyclodextrins. The most important parameter for complex formation with hydrophobic compounds or functional groups is their three dimensional form and size (Table 2.1). The driving force is the entropic effect of displacement of water molecules from the cavity (Saenger, 1980). Another possibility is that this water causes a strain on the cyclodextrin ring, which is released after complexation, producing a more stable, lower energy state (Saenger, 1980; Szejtli, 1982).

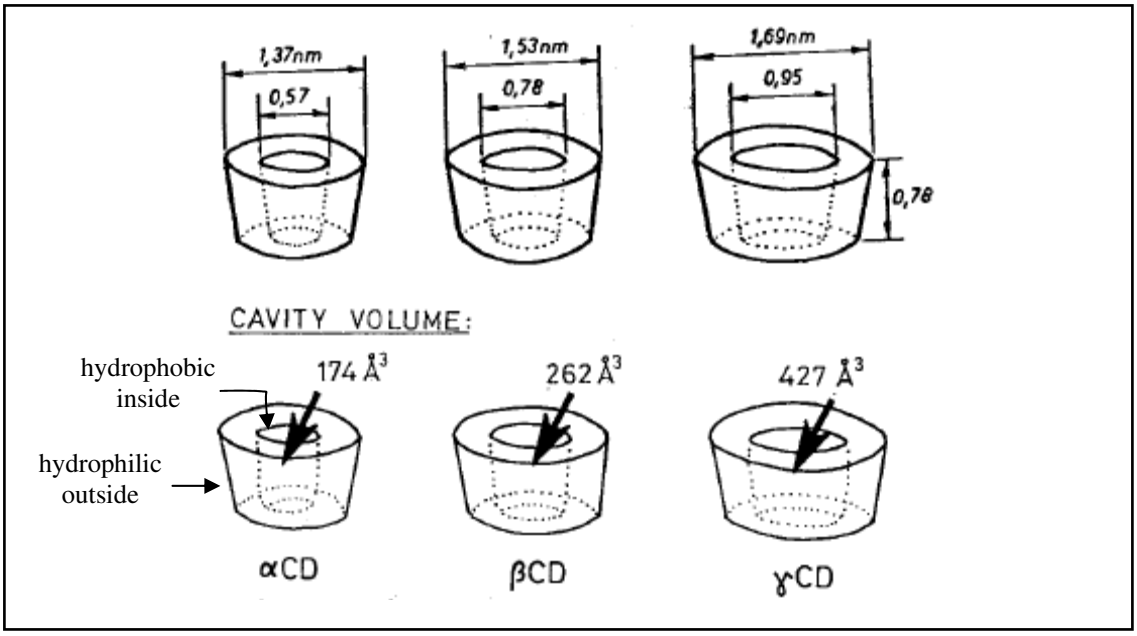

Figure 2.5: Approximate geometric dimension of α-, β-, and γ-cyclodextrin molecules (Szejtli, 1998).

Table 2.1: Properties of α-, β-, and γ-cyclodextrin (Szejtli, 1998).

Properties	**α**	**β**	**γ**
no. of glucose units	6	7	8
mol wt	972	1135	1297
solubility in water, g 100 ml^{-1} at room temperature	14.5	1.82	23.2
cavity diameter, Å	4.7-5.3	6.0-6.5	7.5-8.3
height of torus, Å	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
diameter of outer periphery, Å	14.6 ± 0.4	15.4 ± 0.4	17.5 ± 0.4
approx volume of cavity, $Å^3$	174	262	427
approx cavity volume in 1 mol CD (ml)	104	157	256
in 1 g CD (ml)	0.10	0.14	0.20
crystal forms (from water)	hexagonal plates	monoclinic parallelograms	quadratic prisms

Administered cyclodextrins are quite resistant to starch degrading enzymes, although they can be degraded at very low rates by α-amylases. α-Cyclodextrin is the slowest, and γ-cyclodextrin is the fastest degradable compound, due to their differences in size and flexibility. Degradation is not performed by saliva or pancreas amylases, but by α-amylases from micro-organisms from the colon flora. Adsorption studies revealed that only 2-4% of cyclodextrins were adsorbed in the small intestines, and that the remainder is degraded and taken up as glucose. This can explain the low toxicity found upon oral administration of cyclodextrins (Duchene, 1987).

2.3.2 Application of cyclodextrins

Since every guest molecule is individually surrounded by a cyclodextrin (derivative) the molecule is micro-encapsulated from a microscopical point of view. This can lead to advantageous changes in the chemical and physical properties of the guest molecules. The characteristics of cyclodextrins or their derivatives that make them suitable for applications in analytical chemistry, agriculture, the pharmaceutical field, in food and toilet articles are stabilization of light- or oxygen-sensitive substances, modification of the chemical reactivity of guest molecules, fixation of very volatile substances, improvement of solubility of substances, modification of liquid substances to powders, protection against degradation of substances by microorganisms, masking of ill smell and taste, masking pigments or the color of substances and catalytic activity of cyclodextrins with guest molecules.

2.3.2.1 Analytical chemistry

Cyclodextrins are used for the separation of enantiomers by High Performance Liquid Chromatography (HPLC) or Gas Chromatography (GC). The stationary phases of these columns contain immobilized cyclodextrins or derived supra molecular architectures. Other analytical applications can be found in spectroscopic analysis. In Nuclear Magnetic Resonance (NMR) studies they can act as chiral shift agents and in Circular Dichroism as selective (chiral) agents altering spectra.

In some specific cases cyclodextrin-inclusion complexes can be employed as enzyme-substrate complexes. Because these selective complexes facilitate specific steric attack of the organic inclusion compound, isomerization of the products can be accelerated. Another example of high catalytic activity is provided by a β-cyclodextrin-dinicotinamide derivative which catalyzes the reduction of a number of quinones with enzyme like reaction kinetics (Saenger, 1980).